

Investigation of consequences of chronic neuroinflammation in the cerebellum and brainstem
regions of young rats

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Abstract

In neurodegenerative diseases like Alzheimer's (AD) and Parkinson's (PD), specific areas in the brain degenerate, producing disabling physical and mental symptoms. A major factor of the degeneration is brain inflammation. A recent theory suggests that certain regions in the brainstem could be vulnerable and become negatively affected first in neurodegenerative diseases; however this theory has yet to be extensively explored. I predicted that there would be an elevated amount of inflammation, measured by the number of activated microglia, in the cerebellum because of vulnerability and consequences of surgery. I also predicted regions in the brainstem such as the raphe nucleus (RN) and the locus coeruleus (LC) would be damaged based on previous observations in humans with neurodegenerative diseases. An animal model of neuroinflammation was used to reproduce the pathology of a neurodegenerative disease in order to examine the cerebellum and brainstem. These regions have not yet been studied in this model. An animal model of neuroinflammation is surgically created by diffusing consistent, low-levels of lipopolysaccharide (LPS) into the 4th ventricle of a rat. Histological processes and behavioral tests were conducted. The results show that rats exposed to LPS have more activated microglia in the cerebellum and brainstem than the controls. Rats exposed to LPS have motor impairment and perform more poorly on a hanging task than control rats. The condition of the RN and the LC was qualitatively deduced and the nuclei have fewer neurotransmitters and more activated microglia in the rats exposed to LPS. Inflammation could have negatively affected the condition of those nuclei. Abnormalities of the RN and LC have also been found in humans with AD or PD, thus indicating that the animal model of neuroinflammation I chose for study reproduces important pathological aspects of neurodegenerative diseases and would be useful for future investigations of potential drug treatments.

Introduction

Brain inflammation is a major factor of age-related diseases like AD and PD.

Components of these diseases can be replicated by surgically diffusing an inflammation-inducing material (LPS) into a young rat's brain to study and test treatments. My project included examining the cerebella and brainstems of the rats chronically infused with LPS in order to investigate 1) the degree of neuroinflammation in the cerebellum and 2) the condition of specific nuclei in the brainstem, the locus coeruleus (LC) and the raphe nucleus (RN) (See figure 1). This project was carried out to make further discoveries about an animal model of inflammation and to make contributions to the knowledge base of treatment for those with debilitating neurodegenerative diseases.

The pathology of neurodegenerative diseases like AD and PD is extensive. Patients with AD are confused and have impaired memory. Its main pathology is neurofibrillary tangles and senile plaques in the brain, which can only be diagnosed post-mortem (Freberg, 2006). PD is defined by neuronal loss, specifically an 80% or more death of dopamine neurons in the substantia nigra. Once this 80% threshold is reached, patients begin showing motor deficits like facial freezing and involuntary tremors (Freberg, 2006). Both of these diseases have other major symptoms, such as depression and poor sleep quality. While dopamine loss is the main pathology of PD and plaques and tangles define AD, brain inflammation is also a major factor in both. It is known that neuroinflammatory processes contribute to neurodegenerative diseases (Hirsch & Hunot, 2009, Gasparini, 2004). Neuroinflammation could just be a consequence of neuronal changes or degeneration, or neuroinflammatory processes might be a main cause of disease (Hirsch & Hunot 2009).

The amount of inflammation can be measured by the number of inflammatory processes, specifically in the quantity of activated microglia. Activated microglia are widely considered to play a pivotal role in inflammation (Rogers, 2008). I define the degree of neuroinflammation by the amount of activated microglia in this project.

This animal model of inflammation was created in young rats through brain surgery with the intention of testing the behavioral and neurochemical consequences of chronic neuroinflammation. A cannula, a thin metal tube, is implanted in the fourth ventricle in the cerebellum in order to chronically infuse the brain with LPS or artificial cerebral spinal fluid (aCSF) in control rats. The LPS or aCSF is slowly diffused into the 4th ventricle for 2, 4, or 8 weeks. The verification of neuroinflammation is represented by an increased number of activated microglia in different areas throughout the brain. Quantifying the degree of activated microglia in the cerebellum is crucial to understand the outcome of neurosurgery. For instance, if potential damage is sustained from surgery, it could impact behavioral testing in the form of motor deficits (considering the role of the cerebellum). Qualitatively analyzing the condition of the LC and RN is also important because of the nuclei's role in the pathology of AD and PD in human subjects. An autopsy study done in 1987 concluded that all 20 examined patients with AD, PD, or a combination diagnosis of AD and PD had abnormal LCs and RNs (D'Amato). The LC and RN are vulnerable to damage in neurodegenerative diseases and this damage could be attributable to sleep problems and depression common in AD and PD.

The function of the LC and RN is crucial for an optimal sleep and arousal system. The LC is the main source of noradrenaline for the brain and RN is the main source of serotonin for the brain. Both nuclei are involved in the regulation of arousal and wakefulness (Freberg, 2006). The LC plays a major role in any process which requires the noradrenaline neurotransmitter,

including attention and stress. The RN is important to sleep regulation and also the pathology of depression. A type of prescription anti-depressant called Selective Serotonin Reuptake Inhibitors (SSRIs) acts on serotonin in the RN by blocking the reuptake of serotonin with the purpose of reducing the symptoms of depression. Depression is a major symptom of AD and PD (National Institute on Aging, 2008, Mayo Clinic, 2010). If the condition of the LC and RN is compromised, neurotransmitters noradrenaline and serotonin could be fewer and perform less optimally. The loss of noradrenergic LC neurons in AD has been known, but LC loss is now suspected to be occurring limitedly in PD (Galea et al, 2002). This cell loss and subsequent poor condition could be attributable to the presence of activated microglia in those areas.

The evidence shows that abnormalities in the LC and RN occur in patients who have clinical AD or PD. However, the development of inflammation in the LC and RN could possibly occur before the onset of the neurodegenerative disease. It's theorized that inflammation occurs first in the pons region of the brainstem, which includes the RN and LC (Szot et al, 2009). The foundation for this theory is the likely relationship between the LC and RN and the progression of PD. This relationship can be exemplified in the link between REM Behavior Disorder (RBD) and PD. RBD consists of sporadic physical movements during REM sleep and this condition of abnormal sleep, most likely involving the LC and RN, could be a warning sign for an increased risk of developing PD (Thorpy & Adler, 2005).

Patients with RBD normally produce violent movements in their sleep due to acting out a vivid dream, but they often report a precursory phase which precedes the onset of RBD by months or years and includes talking or limb movements in sleep (Thorpy & Adler, 2005). RBD involves destruction of brainstem structures responsible for the state of paralysis during REM

sleep. The serotonergic structures (RN) and noradrenergic structures (LC) suffer destruction or reduced activity in RBD.

There is also a positive correlation between RBD and the risk of developing PD. There is data to support that RBD may be the “sole heralding manifestation” of eventual Parkinson’s disease in older men (Thorpy & Adler, 2005). In a group of 29 male patients 50 years and older, 11 out of 13 (85%) patients developed PD after RBD was diagnosed (Thorpy & Adler, 2005). People who have abnormal REM sleep features like RBD are more likely to develop PD. This increased risk could be because of the presence of inflammation damaging the LC and RN before it affects the substantia nigra and the patient is subsequently diagnosed with PD.

The principle results show that rats chronically infused with LPS had more activated microglia in the cerebellum and the RN and LC were also in poorer condition. We can determine that the LPS rats have more neuroinflammation by the increased amount of activated microglia. The presence of activated microglia correlates with poorer performance in rearing and hanging. Shorter hanging times in the hanging task and rearing durations both significantly correlated with the high rate of activated microglia, thus indicating that the rats with inflammation in the cerebellum and brainstem had motor deficits. The RN and LC were also examined and both nuclei in LPS rats appear to be in poorer condition with fewer, more scattered neurons and a greater amount of activated microglia surrounding the area. We believe that the presence of activated microglia is leading to neuronal destruction and death in these nuclei.

The overall conclusion is that chronic neuroinflammation negatively impacts the cerebellum and brainstem in this animal model of inflammation. The diffusion of LPS into the 4th ventricle affects vulnerable areas of the brainstem, the LC and RN. Damage to the cerebellum is most likely a consequence of surgery because there is some activated microglia present in the

cerebellum of the control rats which were infused with aCSF. There are implications for this study in terms of future research involving the LC and RN. Implications of neurodegenerative diseases in humans and prevention against neuroinflammation are also discussed.



Figure 1. Areas of interest in the cerebellum and brainstem of a rat.

The diagram (left) shows the area of the cannula tract and also the areas of the examined nuclei: locus coeruleus and raphe nucleus. The photomicrograph (right) shows the actual tissue and the corresponding areas.

Methods

Subjects and surgical procedures

Thirty-seven 3-month old male F-344 rats (Harlan Sprague-Dawley, Indianapolis, IN) were individually housed in Plexiglas cages with freely available food and water. The rats were maintained on a 12/12 hour light-dark cycle in a temperature-controlled room (22 C). All of the rats were given health checks and handled for 3 days before behavioral testing. They were also given 1 week to adapt to their new environment without any interference. After that week, the rats underwent surgery to implant a cannula into the 4th ventricle of the brain in order to

chronically infuse the brain with a low dose of either artificial cerebrospinal fluid (aCSF, n=11) or lipopolysaccharide (Sigma, St. Louis, MO, USA E.coli, serotype 055:B5, TCA extraction, 1.0 ug/ul dissolved in aCSF, n=26). LPS is gram-negative bacteria which will produce inflammation over a long-term exposure. The cannula, which is implanted through the cerebellum and dispenses into the 4th ventricle, is connected (via Tygon tubing, 0.06 O.D.) to an osmotic minipump (Alzet model #2004, to deliver 0.25 ul/h; Direct Corp., Cupertino, CA, USA). The aCSF vehicle contained (in mM) 140 NaCl; 3.0 KCl; 2.5 CaCl₂; 1.0 MgCl₂; 1.2 Na₂HPO₄, adjusted to pH 7.4. The research was conducted under the supervision, and with the approval, of the University of Ohio State University Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering and to reduce the number of animal used to a reasonable minimum regarding statistical constraints.

Behavioral testing

The rats were infused with LPS or aCSF for 2, 4, or 8 weeks. At the end of the exposure, the rats underwent behavioral testing: hanging task and open field. The hanging task measures how long the rats can hang on a bar and tests for motor coordination. A wire bar was covered by rubber tubing and secured over a plastic box, with more than 3 inches from each side. The rat was gently placed on the bar so that the rat was hanging on with all four feet and then timed until the rat fell into the box onto several towels. The first 2 timed attempts were for practice only. The final 3 trials were counted. This task aims to test the rat's ability to coordinate between the front and back legs enough to hold onto the bar for a maximum of 60 seconds.

The task of open field requires the researcher to observe the rats' behavior in a novel environment. A researcher monitored behavior and recorded the frequency of boli, urine, sleep,

and the duration of freezing and rearing on their back legs. The purpose of open field is to test for gross motor impairment, anxiety, and rearing frequency and duration. Open field is conducted in a dry 170cm diameter tub. Bedding from a few of the rats' cages was dispersed on the bottom of the tub to discourage the rats from being attracted to one spot because of the scent of another animal. One rat at a time was gently positioned at a specific place in the dry tub and observed for 15 minutes. The actions of each rat were observed and recorded.

Histological procedures

Immediately after behavior testing was completed, each rat was deeply anesthetized with isoflurane and sacrificed by transcardiac perfusion of the brain. Cold saline containing 1 U/ml heparin was infused for 8 minutes, followed by 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4 for 12 minutes. The harvested brain was post-fixed in 4% paraformaldehyde overnight at 4 °C then changed to phosphate buffer saline (PBS 1x), pH 7.4 for long-term storage at 4 °C.

The cerebellum and brainstem were separated from the rest of the brain and stored in PBS 1x solution at 4 °C. The cerebellum was cut at its posterior with a brain matrix into a 4 ml section. The vibratome was set (speed 4 -6, frequency 10, feed 40 um) and slices stored in a 24 well plate (each well filled with .5 ml of antifreeze). The tissue was organized by 5 pieces of tissue in succession per each well. Tissue was stored at -70 °C until used.

Immunohistochemical staining

Six slices of brain tissue per rat were immunohistochemically stained. The first set consisted of 3 pieces of tissue containing the best sample of the LC. The second set consisted of 3 pieces of tissue per rat with the RN present. The LC is located posteriorly from the RN so 2

sets of tissue were used to provide the best example of each. Tissue with the 4th ventricle at its most prominent point was selected because the RN is located below it. Tissue from 2 wells previous from the 4th ventricle tissue was selected for the LC sample.

The tissue with the 4th ventricle at its most prominent was immunohistochemically with tryptophan hydroxylase (TrypH) for serotonin, tyrosine hydroxylase (TH) for norepinephrine and OX-6 for activated microglia. After the tissue was rinsed with PBS 1x, quenched for endogenous peroxidase activity in 0.3% H₂O₂ in 50% methanol and blocked for nonspecific binding in 5% normal goat serum, primary antibodies to tyrosine hydroxylase (final dilution 1:750, Chemicon) and tryptophan hydroxylase (final dilution 1:100, BD PharMingen) were mixed and applied to the free-floating tissue for 48 hours at 4°C. The tissue was then rinsed with PBS tween, incubated for 2.5 hours with secondary antibodies biotinylated anti-rabbit (final dilution 1:200, Vector) and biotinylated anti-sheep (final dilution 1:200, Vector). Sections were incubated for 2 hours (22 °C) with avidin-biotinylated horseradish peroxidase (Vectastain, ABC kit, Vector, Burlingame, CA). The reaction was stopped by washing the tissue with PBS. The tissue was incubated with SG Blue working solution as chromogen for 2-3 minutes and the reaction was stopped by rinsing with PBS.

Next, the tissue was stained for activated microglia with primary antibody Ox-6 to label positive MHC-II receptors. The tissue was incubated in Ox-6 (final dilution 1:200, BD PharMingen) overnight at 4 °C on the shaker. After it was rinsed with PBS the next day, the tissue was incubated in the secondary antibody anti-mouse (final dilution 1:200, Vector) for 1.5 hours at 22 °C. The tissue was rinsed with PBS and the sections were incubated for 1 hour (22 °C) with avidin-biotinylated horseradish peroxidase (Vectastain, ABC kit, Vector, Burlingame, CA). After rinsing again in PBS tween, the sections were incubated with 0.05% 3,3 -

diaminobenzidine tetrahydrochloride (DAB, Vector, Burlingame, CA) as chromogen for 20 minutes. Finally, the tissue was mounted on gel-coated slides, air-dried, dehydrated and coverslipped with cyto seal (Allan Scientific, Kalamazoo, MI) mounting medium.

The tissue with the best sample of LC was stained for TH to label norepinephrine neurons and for OX-6 to label activated microglia. The procedure to stain this set of tissue is the same as the above except TrypH and its secondary antibody (biotinylated anti-sheep) are excluded because there are no serotonin neurotransmitters in this region.

Image acquisition (confocal microscopy) and Data Collection

The mounted and coverslipped tissue was image acquisitioned using a Nikon microscope. The photomicrographs were inspected at a high magnification for activated microglia surrounding damaged neurons. The LC and RN were qualitatively examined to determine the condition of the neurons and compare the appearances of these nuclei between the control and LPS rats.

A quantitative analysis determined the amount of activated microglia in the cerebellum and brainstem tissue. Multiple selections of tissue for each rat were selected for analysis. The first set of sample tissue (with the 4th ventricle and RN) showed evidence of the completed neurosurgery because of the cannula tract. Each individual slice was given a rating between 0 (no activated microglia) and 6 (excessive activated microglia). The individual ratings were averaged to give each rat one overall activated microglia rating.

The density of activated microglia for a set area of ventral cerebellum (below the 4th ventricle and above the raphe nucleus) was calculated by ImageJ. The data were compared

between the control rats to determine if there was more or less inflammation damage in the cerebellum and brainstem.

Results

Rats chronically infused with LPS had more activated microglia in the cerebellum and brainstem compared to the control rats ($f(1,5)=16.55$, $p<.01$) (See figure 2 and 3). A quantitative analysis was calculated by me and Holly Brothers, M.A., to give each rat an appropriate 0-6 rating based on the amount of activated microglia. Brothers separately and blindly rated the amount of activated microglia for each individual sample of tissue using the same scale. The averaged overall activated microglia rating Brothers assigned to each rat correlated with my ratings ($r_s=0.801$, $n=35$, $p=0.0$).

The same photomicrographs of cerebellum and brainstem were also analyzed densitometrically with ImageJ and averaged overall for each rat once again. This densitometry rating also correlated with my averaged activated microglia rating of 0 – 6 ($r_s=0.67$, $n=30$, $p=0.0$).

The rats underwent behavioral testing after 2, 4, or 8 weeks of infusion of LPS or aCSF. The behavioral tests included hanging and open field. There was a significant difference between the LPS and aCSF groups (4 and 8 weeks) in hanging times during the hanging task ($f(1,19)=27.87$, $p<.01$) (see figure 4). There were correlations between a greater activated microglia rating and poor performance in rearing during open field and also in hanging. The more activated microglia in the cerebellum and brainstem of a rat, the fewer times the animal reared during open field ($r_p=-0.55$, $n=22$, $p<0.01$). The rats with more activated microglia also had less total rearing durations ($r_s=-0.63$, $n=22$, $p<0.01$). The higher activated microglia rating in the cerebellum is negatively correlated with the rat's ability to hang on a bar during the hanging

task ($r_s = -.49$, $n = 23$, $p < .02$) (see figure 5). A rat with a greater amount of activated microglia is more likely to perform worse on the hanging task and thus have a shorter hanging time.

The condition of the LC and RN was qualitatively analyzed by observing changes between the photomicrographs of LPS and control rats in 2 different sets of tissue. The LC appeared to have more activated microglia around the cluster of norepinephrine neurons in LPS rats (see figure 6). The neurons also appeared to be more scattered across a greater distance than the neurons in the control rats. The RN likewise had more activated microglia amongst the serotonin neurons in the rats infused with LPS (see figure 7). The serotonin neurons in the RN appeared to be fewer in the LPS rats. It is possible that the activated microglia is having negative effects on the neurons and causing cell destruction and death.

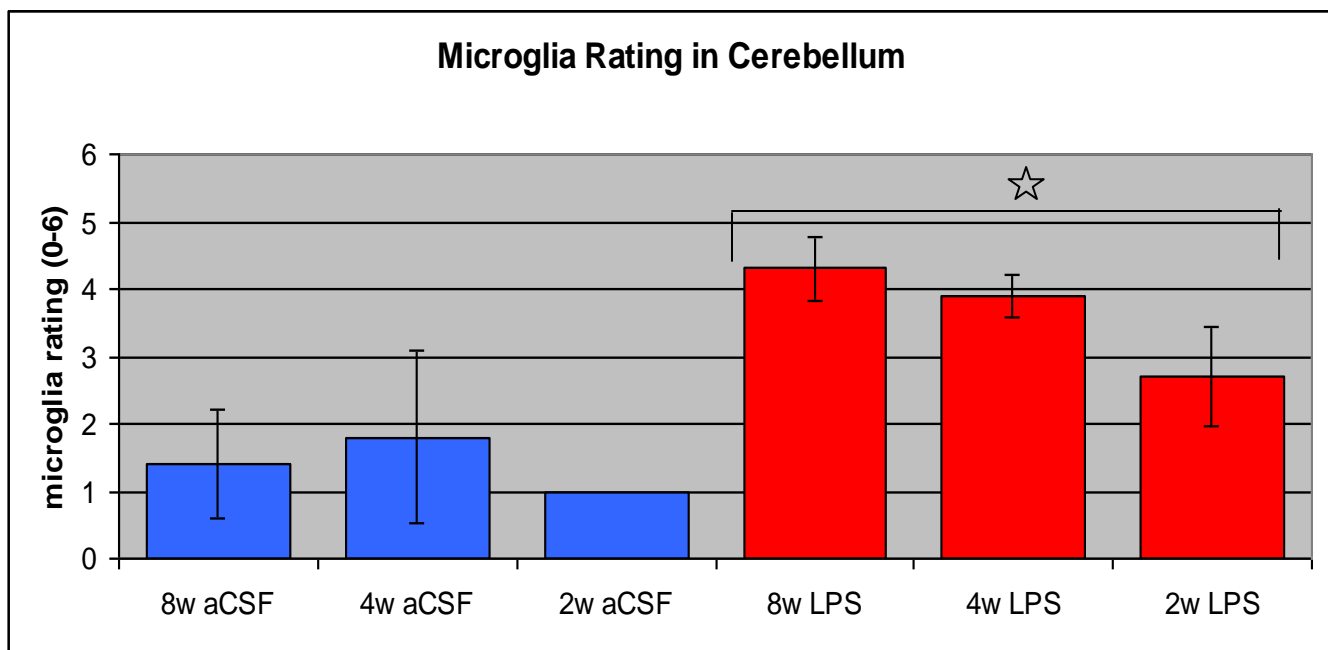


Figure 2. Activated microglia rating in cerebellum.

Rats infused with LPS (in red) had more activated microglia overall than controls (blue) ($p < .01$).

Activated microglia in the cerebellum and brainstem



aCSF



LPS

Figure 3. Activated microglia in cerebellum and brainstem tissue

Typical amounts of activated microglia in an aCSF rat (left) and rat infused with LPS (right). Animals treated with LPS had more activated microglia overall. Activated microglia are the brown cells in this selected tissue.

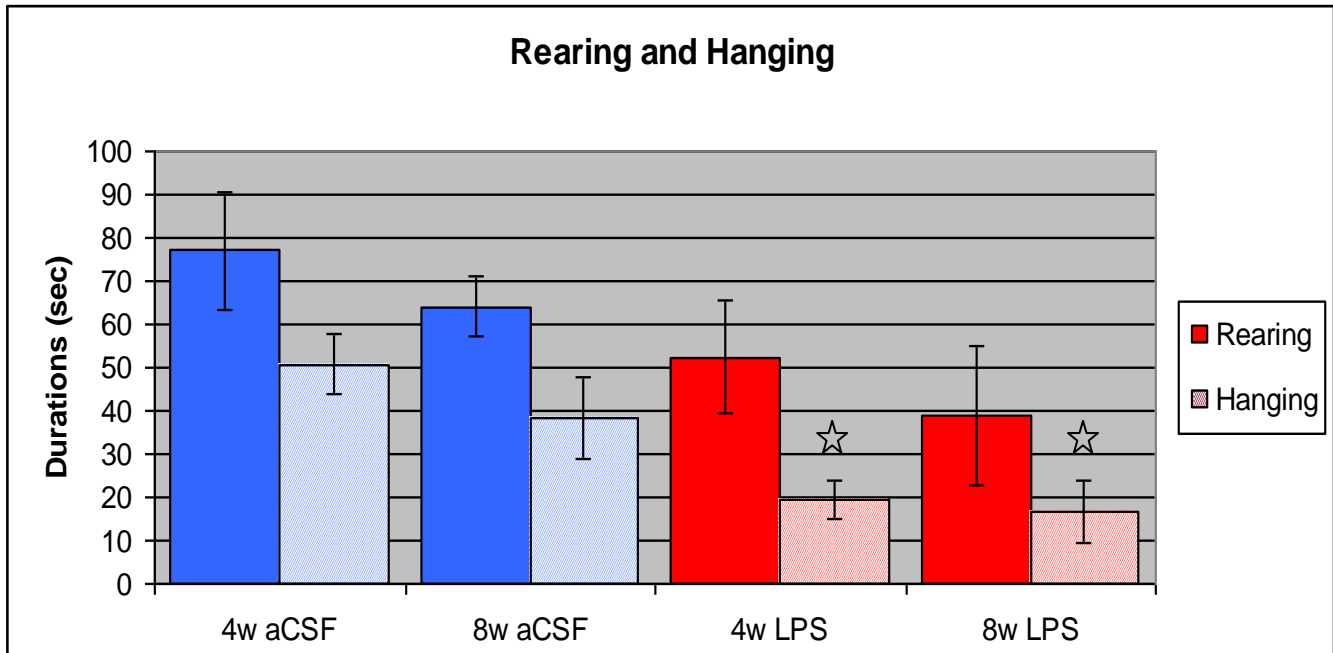


Figure 4. Rearing and Hanging.

The rats infused with LPS had significantly less hanging times than aCSF rats ($p < .01$). Both hanging times and rearing times negatively correlated with activated microglia in the cerebellum and brainstem of rats.

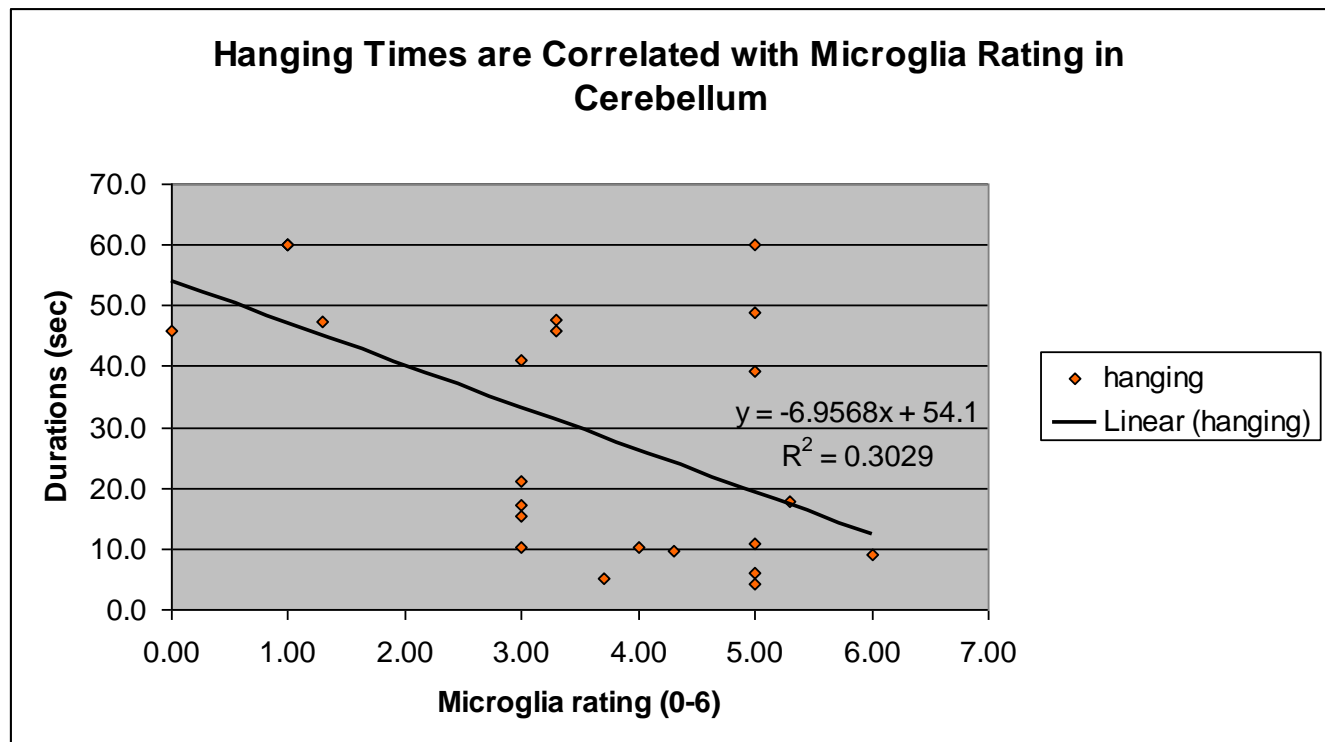
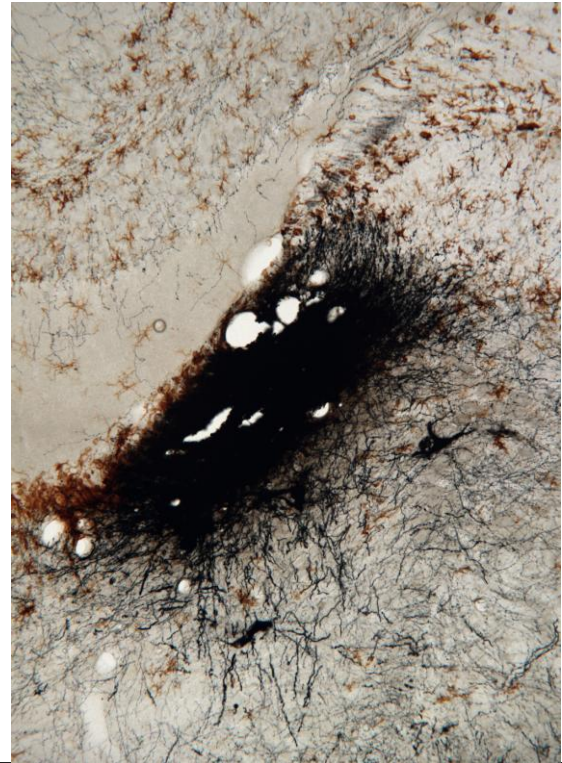
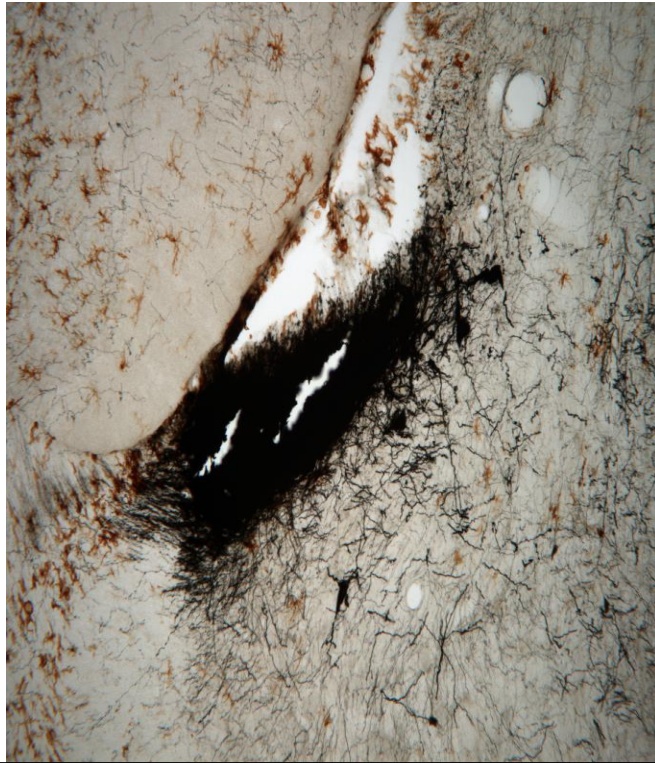


Figure 5. Hanging times correlated with amount of activated microglia.

Rats treated with LPS had a reduced latency to fall in a hanging task that correlated with the amount of activated microglia in the cerebellum ($p < .02$).

Locus coeruleus

aCSF



LPS

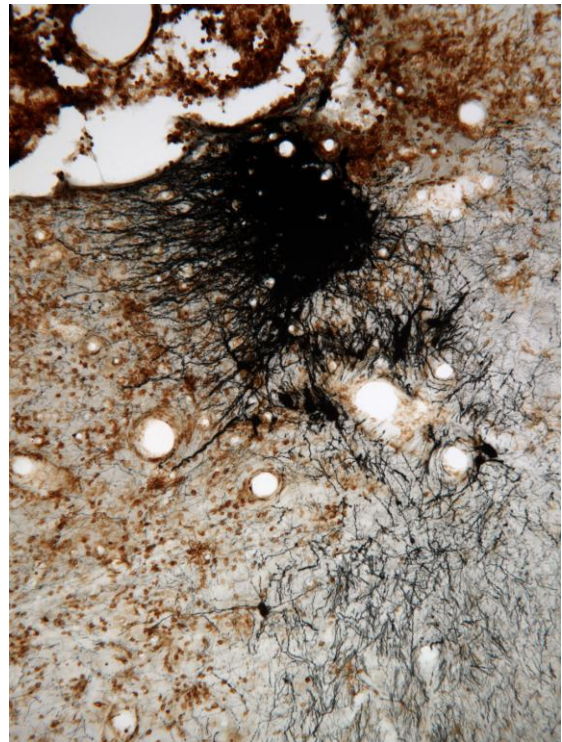
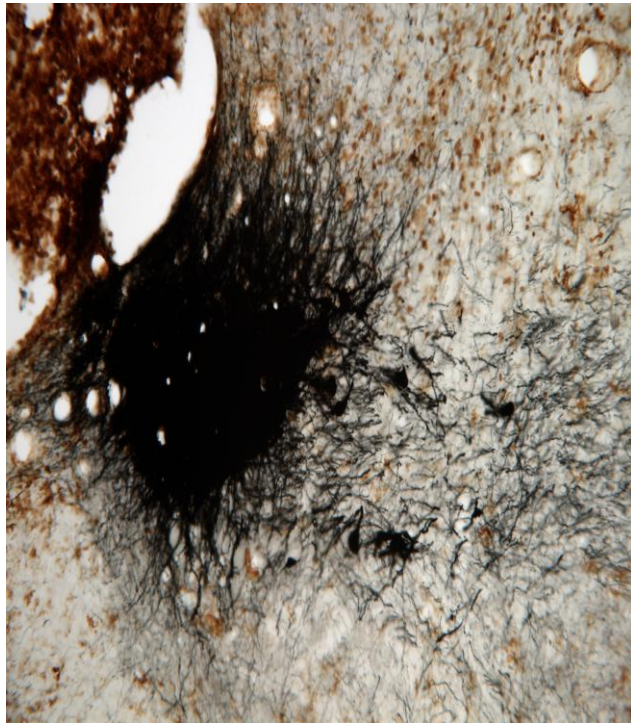


Figure 6. Examples of LC in aCSF and LPS rats.

Norepinephrine neurons in the LC appear more scattered in LPS-treated rats (bottom), possibly due to interaction with the surrounding activated microglia.

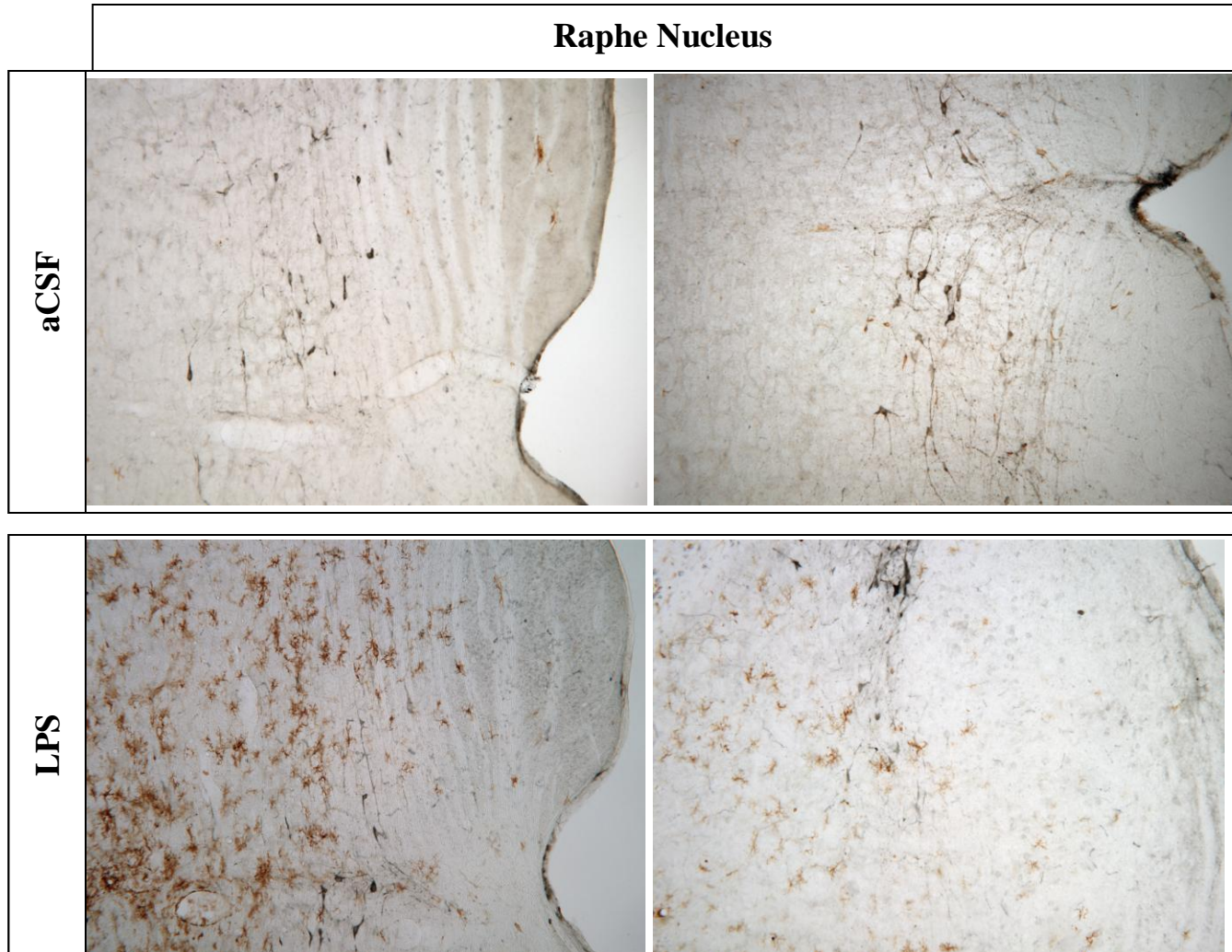


Figure 7. Examples of RN in aCSF and LPS rats.

Serotonin neurons in the RN of LPS treated-rats (bottom) appear bent and twisted possibly as a result from presence of activated microglia.

Discussion

The overall conclusion from this experiment is that chronic neuroinflammation has negative effects on the LC, RN and cerebellum in an animal model of inflammation.

Neuroinflammation in the brainstem affects the LC and RN biochemically and neuroinflammation in the cerebellum correlates with less optimal performance in rats. Such evidence in this analogue study creates implications for humans and the risk of neurodegenerative diseases.

Studies including those involving a genetic focus and epidemiological analysis of humans indicate that neuroinflammatory processes can be risk factors for PD (Hirsch & Hunot 2009). Humans could reduce their risk of developing a neurodegenerative disease by leading an anti-inflammatory lifestyle. Practicing a healthy lifestyle and making choices such as drinking coffee could help protect a person from chronic neuroinflammation (Ross et al, 2000). Another way to protect against AD is to take ibuprofen over a lifetime. It is an anti-inflammatory medication which crosses the blood brain barrier and epidemiological studies have indicated that prolonged use of non-steroidal anti-inflammatory drugs (NSAIDs) like ibuprofen decreases the risk of AD, delays dementia onset and reduces the severity of cognitive symptoms (Gasparini et al).

The cerebellum, LC and RN have not yet been extensively examined in this animal model of inflammation because the main areas of study are the substantia nigra for PD and the hippocampus for AD. However, I predict that in future research of neurodegenerative diseases the LC and RN will be recognized as a vital part of the pathology and that will warrant further study and also pharmacological manipulation of the area.

Examples of valuable future studies would be monitoring the sleep patterns of the animals and also the condition of the LC and RN. These nuclei are highly involved in the

regulation of the sleep and wake arousal system; therefore it would be a good idea to determine if inflammation in the LC and RN would produce sleep disturbances in the rats like what would most likely happen to humans if these areas sustained damage. It would also be beneficial to investigate pharmacological effects on the LC and RN to see if the nuclei could be protected from the neuroinflammation. The LC and RN may be a reliable and valid area to examine for damage and potential treatments.

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